IN THE UNITED STATES PATENT AND TRADEMERK OFFICE

In re Patent Application of
A. Dömling

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For: TUBULYSIN CONJUGATES Examiner: S.R. Gudibande

Commissioner for Patents
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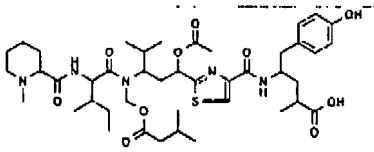
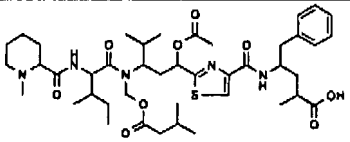
DECLARATION UNDER 37 CFR 1.132

I, Alexander Dömling, declare as follows:

1. I am the Inventor on the above-identified patent application (referred to below as "the patent application"). I earned a Ph.D. degree in Chemistry from the Technical University in Munich in 1993. Subsequently, I was Vice-President Chemistry of Morphochem AG until 2003 and then in 2004 co-founded R&D Biopharmaceuticals GmbH. I am currently Associate Professor of Pharmacology at the University of Pittsburgh.

2. The following experimental work as detailed in paragraph 3 below and in the enclosed poster hand-out were conducted by me or persons working under my direction.

3. The tubulysin compounds identified in the table below were tested in an acid phosphatase assay for activity against human cancer cell lines of MCF-7 and KB-V1. The protocol of the acid phosphatase assay was as described in Yang et al., Anal. Biochem. 241 (1996) 103.

No	Structure	Code	MCF-7 IC50 [ng/ml]	KB-V1 IC50 [ng/ml]
1		Tubulysin A	0.7	1.0
2		Tubulysin D	0.5	0.3

4. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing therein.

Date: 30/06/07

Alexander Dömling

Encls.:
- poster hand-out "Preclinical antitumor activity of Polymer,Tubulysin Nanoparticles in Human Colorectal Cancer Xenograft"

Preclinical antitumor activity of Polymer-Tubulysin Nanoparticles in Human Colorectal Cancer Xenograft

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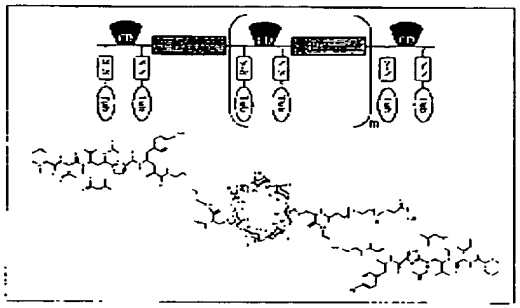
I. Introduction

Polymer-Tubulysin nanoparticles are conjugates of a Tubulysin A (Tub A) derivative and a linear, cyclodextrin-based polymer (CDP). A similar polymer-camptothecin conjugate is in clinical trials now for cancer treatment. Tub A is a naturally occurring tetrapeptide isolated from strains of myxobacteria. It is highly active against multiple cancer cell lines with an IC_{50} in the low nM to pM concentration range. It acts as an antimitotic agent that depolymerizes cell microtubules and triggers apoptosis. The Tub A derivative is covalently attached to CDP through a disulfide linker (Figure 1).

Figure 1: Structure of CDP-S-S-Tub



II. Characterization and Release Studies

The Tub A derivative was incorporated to the polymer backbone with a loading of 12% by weight, as measured by HPLC. The particle size of the parent polymer was measured to be 9-10 nm while CDP-S-S-Tub self-assembled into nanoparticles with a particle size of 127 nm. The solubility of Tub A in water was determined to be 0.1 mg/mL at a neutral pH while that of CDP-S-S-Tub was found to be 100 times higher.

Release studies was performed by incubating CDP-S-S-Tub in both PBS and human plasma. The conjugate was found to be stable in both conditions for greater than 72 h at 37°C.

III. In vitro Studies

The antiproliferative activity of CDP-S-S-Tub was evaluated in vitro in multiple human cancer cell lines (Table 1). The data shows that the conjugate maintains high antiproliferative activity.

Table 1: IC_{50} studies

	IC_{50} (nM)		
Cell lines	CDP-S-S-Tub	Tub A	Tub-S-S
NCI-H1299 (lung) cells	23.7	2.8	N/A
HT-29 (colon) cells	4.9	1.3	4.4
A2780 (ovarian) cells	1.3	2.4	N/A

IV. MTD Studies

The maximum tolerated dose (MTD) of CDP-S-S-Tub was determined in nude mice and found to be between 3 to 10 mg/kg (in Tub equivalents) whereas that of Tub A was not yet established due to 100% mortality at a dose of 0.3 mg/kg (Table 2).

Table 2: MTD studies [a]

Group	n	Treatment agent	mg/kg	Mean BW Nadir[b]	# of TR[c]	Avg Day of TR
1	4	CDP-S-S-Tub	10	(-)23.4% Day 5	4	7
2	4	CDP-S-S-Tub	3	(-)8.7% Day 11	0	N/A
3	4	CDP-S-S-Tub	1	(-)0.4% Day 2	0	N/A
4	4	Tub A	3	(-)9.7% Day2	4	3
5	4	Tub A	1	(-)15.7% Day 3	4	4
6	4	Tub A	0.3	(-)18.9% Day 8	4	9

[a] all mice were treated with schedule qwkx3 using iv injection, [b] Nadir: the lowest point.
[c] TR: treatment related deaths

V. Efficacy Studies

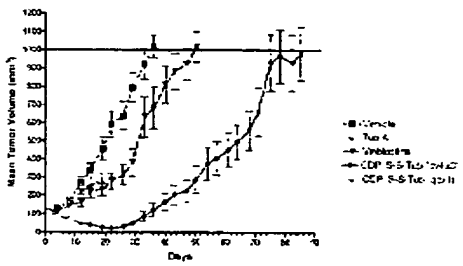
Preclinical efficacy was evaluated in nude mice bearing subcutaneously implanted HT-29 colorectal xenografts. Treatment with CDP-S-S-Tub was well tolerated, with no mortality and significant antitumor effect. It was better tolerated than vinblastine and Tub A. Treatment with CDP-S-S-Tub resulted in higher number of regressions and significant increase in tumor growth delay compared to Vinblastine. Treatment with Tub A was proven to be toxic for the mice, causing 50% mortality and 26.8% maximum body weight loss on day 26 (Table 3 and Graph 1 & 2).

Table 3. Summary of antitumor activity (Endpoint: TV = 1000 mm³ or Day 90, whichever comes first)

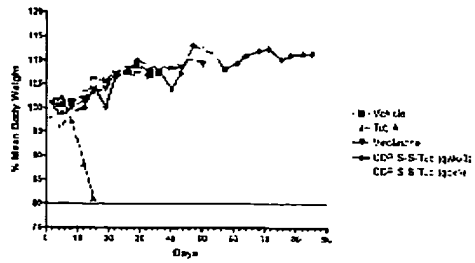
		Treatment Regimen[a]							Statistical Significance[f]			Regressions			Deaths
Group	n	Agent	mg/kg	Schedule	MTV(n), Day 90[e]	BW Nadir	Median TTE	TC	%TGD	vs G1	vs G4	vs G5	PR	CR	TFS	TR
1[b]	10	Vehicle[c]	–	qwkx3	–	–	33.65	–	–	–	***	***	0	0	0	0
2	10	Tub A	0.1	qwkx3	–	-25.80%	34.46	0.8	2.42	ns	ns	ns	0	0	0	5
3	10	Vinblastine	4	qwkx3	–	-3.60%	45.05	11.4	33.89	**	***	ns	0	0	0	0
4	10	CDP-S-S-Tub	3[d]	qwkx3	700.0 [3]	-2.20%	73.55	39.9	118.57	***	–	***	6	3	1	0
5	10	CDP-S-S-Tub	3[d]	qdx1	–	-2.90%	56.92	23.27	69.15	***	***	–	0	0	0	0

[a] all mice were treated using iv injection, [b] control group, [c] vehicle: 10% DMSO: 1% Tween 80: 89% Saline; [d] active Tub dose equivalents; [e] MTV(n): median tumor volume (mm^3) for the number of animals on the day of TGD analysis (excludes animals with tumor volume at endpoint), [f] ns=not evaluable, ns=non-significant, **=0.001<P≤0.01, ***=P≤0.001

Graph 1. Tumor Growth Delay in HT-29 xenograft implanted mice



Graph 2: Body Weight Loss in HT-29 xenograft implanted mice



VI. Conclusion

- A polymer-tubulysin conjugate CDP-S-S-Tub, was synthesized and found to be highly soluble in water and stable in both PBS and human plasma.
- The conjugate showed high antiproliferative activity in multiple human cancer cell lines.
- The MTD of CDP-S-S-Tub was determined to be between 3 to 10 mg/kg while the free drug Tub A, was severely toxic even at 0.1 mg/kg.
- Efficacy studies of CDP-S-S-Tub at 3 mg/kg showed that it was well tolerated and produced substantial antitumor activity during a 90 day study. By contrast, the free drug Tub A, showed excessive toxicity, causing 50% mortality.
- Vinblastine, a vinca alkaloid that inhibits tubulin polymerization by binding to the same binding site as Tub A, was significantly less effective as antitumor agent compared to CDP-S-S-Tub.

VII. Acknowledgments

We thank Piedmont Research Center for performing all of the animal studies.